ENDOTHELIAL DYSFUNCTION AND ARTERIAL STIFFNESS IN CORONARY ARTERY DISEASE

By

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This dissertation is dedicated to:

Shannon, my love and best friend. She is an amazingly beautiful, smart and loving person and I know I'm extremely lucky to have her in my life. It's wonderful to be able to share everything from science to running (when I can get out of bed) with someone I love and respect so much. She means the world to me and I can't wait to see what the future holds for us.

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ENDOTHELIAL DYSFUNCTION AND ARTERIAL STIFFNESS IN CORONARY ARTERY DISEASE

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Endothelial dysfunction (ED) is well documented in both resistance vessels and conduit arteries of coronary artery disease (CAD) patients and has been shown to be a predictor of future cardiovascular events in these patients. The mechanism of ED in CAD appears to be multi-factorial with possible mechanisms including: 1) decreased expression or synthesis of endothelial nitric oxide synthase, and 2) inactivation of nitric oxide (NO) by reactive oxygen species. Exercise-training has been shown to improve endothelial function in CAD but the mechanisms have yet to be elucidated. The objective of this study was to investigate the effects of 12 weeks of standard cardiac rehabilitation on endothelial function, arterial stiffness, oxidative stress and antioxidant defenses, and inflammation in CAD. Twenty CAD patients, 10 exercise trained (ET), 10 control (C), participated in the study. Exercise-training resulted in a significant improvement ($p<0.05$) in brachial artery flow-mediated dilation (FMD) (7.2% at baseline v. 11.2% at 12 weeks) and a significant reduction ($p<0.05$) in indices of arterial stiffness

(augmentation index (AIx) (29.9% at baseline v. 26.2% at 12 weeks) and delay of the reflected wave (Δt) (136.2 ms at baseline v. 144.4 ms at 12 weeks) in the ET group with no change in the C group. Exercise-training also significantly increased ($p<0.05$) plasma nitrate/nitrite (NOx) levels in the ET group (28.6 μmol/L at baseline v. 34.7 μmol/L at 12 weeks). Total plasma 8-isoprostane-$F_{2\alpha}$, a marker of oxidative stress, was significantly reduced (490.5 pg/ml at baseline v. 406.7 pg/ml at 12 weeks, $p<0.05$) and plasma superoxide dismutase activity was significantly increased in the ET group (1.49 U/ml at baseline v. 1.64 U/ml at 12 weeks, $p<0.05$). Finally, C-reactive protein, a marker of inflammation, was significantly reduced (0.276 mg/dl at baseline v. 0.180 mg/dl at 12 weeks, $p<0.05$) in the ET group following exercise-training. Twelve weeks of cardiac rehabilitation improved endothelial function and arterial stiffness possibly through through increased nitric oxide production and reductions in oxidative stress and inflammation. These results suggest that exercise-training may reduce the risk of future cardiovascular events through improvements in endothelial function and arterial stiffness and reductions in oxidative stress and inflammation.

CHAPTER 1
INTRODUCTION

Endothelial dysfunction is a hallmark of coronary artery disease (CAD) and has been shown to be a predictor of future cardiovascular events in these patients (1,2). As opposed to healthy individuals in whom maximal cardiac output is the main determinant of exercise capacity, CAD patients may be limited by myocardial ischemia determined by coronary perfusion and cardiac work. Endothelial dysfunction contributes to ischemia by reducing coronary perfusion and increasing cardiac work through increases in arterial stiffness (3,4). In CAD, endothelial dysfunction appears to be related to decreased nitric oxide (NO) production and a reduction in NO availability. Superoxide anion production is elevated in CAD and may react quickly with NO to decrease its availability (5). Exercise training in healthy individuals and animal models of disease improves endothelial function by increasing antioxidant defenses and through increased nitric oxide (NO) production, thereby altering the vasodilator/vasoconstrictor balance (6,7). However, it is not known whether exercise training has the same effect in CAD. The primary purpose of this study was to investigate the effects of exercise training on endothelial function, arterial stiffness, oxidative stress and antioxidant defenses, and the release of NO in patients with CAD.

We hypothesized that endothelial dysfunction in CAD is the result of a reduction in NO synthesis and an increase in oxidative stress. We propose that 12 weeks of endurance exercise training will have a beneficial effect on the availability of NO in CAD leading to an improved vasodilatory capacity. Additionally, we hypothesize that

exercise training will have a positive effect on arterial stiffness and wave reflection in CAD. There have been only two prior studies that have examined the effect of exercise training on vascular reactivity in CAD patients. The first study examined only adaptation in the coronary circulation (3). In that study, inpatients performed high-intensity exercise for 60 minutes of cycling per day for 4 weeks. They reported improved coronary epicardial and resistance vessel response to acetylcholine and adenosine compared to outpatient controls who received similar pharmacological therapy but led a sedentary lifestyle (3). More recently the effects of 10-weeks of endurance exercise as part of a cardiac rehabilitation program, resulted in improved posterior tibilias flow-mediated dilation (FMD) (8). In healthy individuals, higher aerobic fitness levels have been shown to be associated with lower central arterial stiffness as measured by pulse wave analysis (9), pulse wave velocity (10) and magnetic resonance imaging (MRI) (11) suggesting that large artery arterial stiffness may be influenced by exercise capacity. However, the effects of exercise training on arterial stiffness in CAD have yet to be investigated. Additionally, there have been no studies designed to investigate the effects of exercise training on the production or availability of NO, oxidative stress and antioxidant defenses in CAD.

It is now established that CAD is an inflammatory process and future cardiovascular events can be predicted based on selected inflammatory markers in addition to endothelial function. C-reactive protein (CRP) is the most robust of these markers and interleukin-6 (IL-6) has also shown some promise as a predictor of cardiovascular events (12). Previously, we studied 11 CAD patients participating in standard cardiac rehabilitation. We found a significant decrease in CRP and a trend

($p=0.1$) toward a reduction in IL-6. Prior to studies conducted in our lab, there had only been one cross sectional study investigating the relationship between exercise and CRP (13). In this study physical activity was associated with lower CRP levels (13). The results of these two investigations suggest that exercise training has a beneficial effect on inflammatory mediators. Because CRP has been shown to be an independent predictor of cardiovascular events in both healthy individuals and CAD patients one would hope that an intervention reducing CRP levels would reduce risk for future events as well. A secondary purpose of this study was to investigate the effects of exercise training on inflammation in patients with CAD.

This is the first prospective study to evaluate the effects of endurance exercise as prescribed in cardiac rehabilitation (the standard of care in the United States) on vascular function, oxidative stress and inflammation in patients with documented coronary artery disease (CAD). Thus, this proposal will employ unique experiments that will further the understanding and treatment of CAD. The specific aims of this proposal are as follows:

Specific Aim 1: To measure endothelial function, arterial stiffness and plasma levels of nitrate/nitrite (NOx) in CAD patients before and after 12 weeks of standard cardiac rehabilitation or 12 week control period.

Hypothesis 1: Exercise training will result in improved endothelial function, a reduction in arterial stiffness and an increase in plasma NOx levels in CAD. Rationale: Endothelial dependent function, as assessed by high-resolution ultrasound and plethysmography, is impaired in CAD indicating either a reduction in stimulated NO production or availability (14). As a whole the studies of endothelial function following exercise training have, for the most part, resulted in improvements in endothelial function

in healthy and diseased states (6,15,16). Cross sectional studies have shown that exercise capacity or increased physical activity is associated with reduced arterial stiffness suggesting that exercise training may have a beneficial effect on the arterial system (4). NO production may be reduced in CAD secondary to reduced expression of endothelial nitric oxide synthase (eNOS) (17). Exercise training has been shown to increase eNOS expression in animal studies (18) and exercise training has been shown to be associated with higher levels of plasma nitrate in healthy individuals (19) suggesting that exercise training may improve NO synthesis.

Specific Aim 2: To measure the plasma levels of 8-isoprostane-$F_{2\alpha}$, superoxide dismutase activity, glutathione peroxidase activity and total antioxidant status in CAD patients before and after 12 weeks of standard cardiac rehabilitation or 12 week control period.

Hypothesis 2: Nitric oxide production and availability is decreased in CAD through increased free radical production and inadequate antioxidant defenses. Exercise training will result in a reduction in oxidative stress and an increase in antioxidant defenses. Rationale: Measures of oxidative stress are elevated in CAD and treatment with vitamin C improves endothelial function indicating that oxidative stress may play a role in endothelial dysfunction in this population (20,21). Extracellular SOD levels have been shown to be reduced in CAD and are highly correlated with both flow-mediated dilation and severity of disease (22). In animals exercise training increases levels of EC-SOD (23) suggesting that exercise training may improve antioxidant defenses helping to increase NO availability.

Specific Aim 3: To measure the plasma levels of C-reactive protein (CRP) and interleukin-6 (IL-6) in CAD patients before and after 12 weeks of standard cardiac rehabilitation or 12 week control period.

Hypothesis 3: Inflammation as measured by CRP and IL-6 will be reduced following exercise training in CAD. Rationale: Physical activity has been shown to be associated with lower levels of CRP in a cross-sectional study (13). Additionally, we have previously demonstrated in our laboratory that exercise training reduces CRP levels in 11 CAD patients suggesting that exercise training may reduce inflammation.

CHAPTER 2
REVIEW OF LITERATURE

The endothelium is a monolayer of cells that line blood vessels separating the vessel lumen from vascular smooth muscle cells. The endothelium is a metabolically active organ that produces a variety of vasoactive mediators. An inability of the endothelium to produce vasodilation in response to stimuli and protect blood vessels from atherosclerosis is termed endothelial dysfunction. Endothelial dysfunction is a hallmark of coronary artery disease (CAD) and can result in a reduction of tissue blood flow and an increase in arterial stiffness. This review will focus on normal endothelial function, the possible mechanisms and consequences of endothelial dysfunction with a particular emphasis on CAD, and the possible role of exercise training in improving endothelial function and arterial stiffness.

Endothelium Derived Vasodilators

Nitric Oxide

Furchgott and Zawadzki (24) showed that the presence of vascular endothelial cells was essential for acetylcholine to produce relaxation of isolated rabbit aorta. However, removal of the endothelium did not stop relaxation of the aorta by the addition of glycerol trinitrate. The authors concluded that endothelium dependent relaxation of smooth muscle was mediated by an endogenous substance that was initially named endothelium derived relaxing factor (EDRF). EDRF was subsequently identified as nitric oxide (NO) (25,26).

Endothelium derived NO is synthesized from the amino acid L-arginine by the endothelial form of nitric oxide synthase (eNOS) yielding L-citrulline as a byproduct in two successive reactions as shown in Figure 2-1 (27).

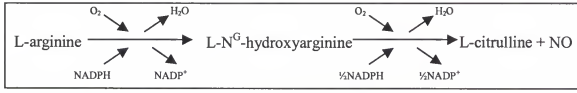

Figure 2-1. Formation of nitric oxide.

In endothelial cells eNOS is constitutively expressed and localized to plasmalemmal caveolae (28). Additionally, eNOS is associated with caveolin, the structural protein within caveolae (29). The association with caveolin inhibits eNOS activity. Agonists or shear stress stimulated increases in intracellular calcium causes the calcium dependent protein calmodulin to disrupt the complex between eNOS and caveolin (30). Therefore eNOS activation is calcium dependent. Another protein-protein interaction involves heat shock protein 90 (Hsp90) facilitation eNOS activity. eNOS complexes with Hsp90 and subsequently results in increased NO production (31). Inhibiting this complex results in increased superoxide production by eNOS (31). Tetrahydrobiopterin (BH_4) is a required cofactor for NO synthesis by eNOS (32). It appears that BH_4 prevents eNOS mediated superoxide production by coupling L-arginine oxidation to NADPH consumption (33). Nitric oxide is labile with a half-life of approximately 10 seconds in vivo (34). It is rapidly oxidized to nitrite and then nitrate by oxygenated hemoglobin before being excreted in the urine (27). Once synthesized NO diffuses across the endothelial cell membrane and into the vascular smooth muscle cells (VSMC) where it interacts with the heme group on soluble guanylate cyclase, leading to an increase enzymatic conversion of guanosine triphosphate (GTP) to cyclic guanosine-

3',5-monophosphate (cGMP) (27). Increased levels of cGMP leads to activation of protein kinase G which phosphorylates calcium regulatory proteins decreasing cytosolic calcium (35). Experiments in which guanylate cyclase was inhibited revealed that NO does have some cGMP independent effects. It appears that NO may cause direct activation of calcium-dependent potassium channels or other ion channels responsible for intracellular calcium homeostasis (36).

Nitric Oxide Release

Basal Release. Vasoconstriction of blood vessels is increased under the conditions of NOS inhibition or when the endothelium has been removed indicating that NO is released under resting conditions from endothelial cells to inhibit contraction of VSMC (37). It appears that basal release of NO occurs due to the fact that resting levels of endothelial cell intracellular calcium are sufficient enough to activate calmodulin (38).

Agonist Stimulated. A variety of chemical substances such as acetylcholine, bradykinin, seratonin and substance P are able to induce NO release and cause endothelium-dependant vasodilation (39). The vasodilation caused by these substances can be prevented using a NOS inhibitor providing evidence that the vasodilation produced by these substances is NO-mediated (39). However, the blockade is not complete suggesting that there may be additional mechanisms responsible for agonist-stimulated vasodilation such as prostaglandins or EDHF (39).

Shear Stress. Physiologic shear stress is an important regulator of NO release from the endothelium. The mechanism of shear stress induced release of NO is complex and involves extremely rapid changes via ion channel activation, phosphorylation of eNOS and a slower increase in eNOS mRNA and protein.

A number of in vitro studies have provided evidence that ion channels, including certain calcium, potassium and chloride channels, open seconds after exposure to shear stress. Application of shear stress to bovine aortic endothelial cells leads to an immediate large increase in intracellular free calcium within one minute (40). This increase in calcium occurs only in response to pulsatile flow and steady flow and leads to an increased production of NO by eNOS (40).

Mechanical activation of eNOS as induced by shear stress also occurs via tyrosine phosphorylation (41). This effect is independent of intracellular calcium concentrations and may lead to translocation of eNOS from the cytosol to the plasma membrane (41). This phosphorylation directly increases eNOS activity at resting calcium concentrations indicating the shear stress-induced activation of eNOS is apparently calcium-independent (42).

Shear stress also stimulates eNOS gene transcription to maintain long-term nitric oxide production. Application of shear stress results in an induction of eNOS mRNA in a dose dependant manner in both bovine and human aortic endothelial cells (43). Additionally, chronic exercise in dogs increases coronary vascular NO production and eNOS gene expression most likely through a hemodynamic mechanism (44).

Prostacyclin

Nitric Oxide is the predominant endothelium derived vasodilator but prostacyclin was discovered as an endothelial derived vasodilator long before NO (45). Shear stress and agonists also lead to the activation of phospholipase A_2 which leads to an increase in arachidonic acid release from phospholipids ultimately leading to an increase in eicosanoid production (35). Prostacyclin is the only ecosanoid that seems to play an important role in endothelial mediated control of vascular tone (46). Flow mediated as

well as agonists stimulated endothelium-dependent vasodilation can be in part inhibited by cyclooxygenase blockers indicating a role for prostacyclin (35).

Endothelium-Derived Hyperpolarizing Factor (EDHF)

Electrophysiological studies of arteries have established that endothelium-dependant hyperpolarization of VSMC is resistant to the combined inhibition of both NOS and cyclooxygenase (47,48). Therefore, a part of endothelium-dependent relaxation is mediated by a substance other than NO and prostacyclin. The hyperpolarization has been attributed to a diffusible endothelium-derived hyperpolarizing factor (EDHF) (49). However, the identity of this substance is still controversial but it appears that EDHF acts by opening potassium channels in VSMC (47).

Endothelium Derived Contracting Factors

Endothelin-1

In the mid 1980's it was shown that the endothelium generated a peptide with long-acting vasoconstrictor action and was subsequently named endothelin (ET) (50). Three isoforms were identified and named ET-1, ET-2, and ET-3 with ET-1 the predominant isoform found in the vasculature (51). Prepro-endothelin is the initial product of ET-1 gene activation which is then shortened to Pro ET-1 or Big ET-1 (50). Big ET-1 is then converted to ET-1 by the membrane bound endothelin-1 converting enzyme (ECE-1) (52). Generation of ET-1 can be increased by various stimuli such as angiotensin II, arginine vasopressin (AVP), free radicals and lipoproteins; its production can be inhibited by nitric oxide, atrial natriuretic peptide and prostaglandins (53).

Vasoconstrictor Prostaglandins

Metabolism of arachidonic acid by cyclooxygenase may lead to the secretion of prostaglandin H_2 the precursor to all prostanoids including thromboxane A_2 (45). Both

prostaglandin H_2 and thromboxane A_2 induce vasoconstriction in VSMC (54). However, under normal conditions prostacyclin is the major metabolite of arachidonic acid and the production of small amounts of vasoconstrictor prostanoids is masked by NO, prostacyclin and EDHF (55).

Oxidative Stress

Endothelial cells release oxygen-derived free radicals in response to various stimuli (55). The potential sources of these radicals in vascular cells are xanthine oxidase, NAD(P)H oxidase and eNOS (56). Nitric oxide and superoxide react in a diffusion limited reaction to form peroxynitrite which is a strong oxidant with only minimal vasodilator activity (57). NO is subsequently no longer available to produce vasodilation of VSMC. Peroxynitrite and other radicals can modify arachidonic acid and LDL to generate isoprostanes independently of cyclooxygenase (58). F_2-isoprostanes, specifically 8-epi-prostaglandin $F_{2\alpha}$, have been used as markers of oxidative stress but have also been shown to have a vasoconstrictor action on VSMC (58). Therefore, the production of oxygen-derived free radicals inactivates NO and produces vasoconstricting isoprostanes.

Endothelial Dysfunction

Endothelial dysfunction is present in atherosclerosis, risk factors for atherosclerosis and vascular diseases. Essentially, endothelial dysfunction is characterized by an impaired bioactivity of endothelial NO. The possible mechanisms of impaired nitric oxide activity include eNOS gene polymorphisms, altered gene expression and mRNA stability, altered signal transduction, decreased substrate and/or cofactor availability and inactivation of NO (32). An exhaustive review of these

mechanisms is not possible here but will subsequently be reviewed in the context of coronary artery disease.

Arterial Stiffness

Increased arterial stiffness and reduced distensibility results in an impaired ability to absorb pulsations from the ejection of the LV (59). These changes are characteristic of aging but are also observed in atherosclerosis and may be the consequence of neurohumoral activation, including sympathetic activation and the renin-angiotensin system, and endothelial dysfunction. Increased arterial stiffness causes the return of the reflected pressure wave to occur during systole and thus increase afterload. The function of the heart is to pump blood through the arterial system to the organs and tissues in an amount sufficient to meet their metabolic needs at rest and during exercise or periods of stress. If the heart is ideally matched to its afterload ventricular/vascular coupling and cardiac efficiency are optimal and myocardial oxygen consumption is minimal (59). The amount of energy and oxygen used by the left ventricle to maintain cardiac output is dependent on the contractile properties of the myocardium and the physical properties of the arterial system. The arterial system constitutes the external afterload placed on the ventricle during contraction and ejection. The load has both static and dynamic components since the ventricle ejects a pulsatile blood flow into a distensible arterial system. The static or resistive component is dependent on blood viscosity and arteriolar caliber, while the dynamic or compliant component is dependent on the elastic properties of the larger arteries and pulse wave reflections (59). Therefore, afterload is composed of peripheral resistance, arterial stiffness and the reflectance of pressure and flow waves. An increase in the pulsatile components of afterload, stiffness and reflectance, causes and

unfavorable mismatch between the ventricle and the arterial system (60-62) increasing myocardial oxygen consumption and decreasing cardiac efficiency (63). These deleterious changes in ventricular/vascular coupling can promote the development of left ventricular hypertrophy and coronary artery disease which often lead to myocardial infarction, coronary artery disease and cardiac arrest (64).

The distensibility of arteries can be estimated from measurements of pressure or flow pulse wave propagation along the vessel wall (59). Bramwell and Hill (65,66) determined arterial elasticity from measurements of pulse wave velocity (PWV) in humans of different ages and found that oxygen consumption and the amount of energy that the ventricle expends per beat varies with the elasticity or stiffness of the arterial system. Increases in arterial stiffness and pulse wave velocity (PWV) in humans cause profound changes in ascending aortic pressure and flow wave contours. These changes are attributed to the timing and intensity of pulse wave reflections from peripheral reflecting sites (59,66,67). Early return of reflected (or backward) pressure waves merge (or sum) with the incident (or forward) pressure wave while reflected flow waves subtract from the incident flow wave (68). The most extensive study of arterial stiffness has occurred in the area of aging. Murgo performed a series of studies of pressure wave contour in the ascending aorta of humans (67,69). These pressure waves are characterized by a well-defined inflection point in the mid-to-late part of systole followed by a secondary wave. This is the result of the reflected pressure wave returning from the periphery. As we age or arterial stiffness increases the reflected wave begins to return earlier in systole resulting in an augmentation of pressure. These changes are amplified in the case of hypertension and other disease with accelerated arterial stiffening. The

reflected wave amplitude is greater and occurs progressively earlier in cases of increased arterial stiffness and can be explained on the basis of PWV and amplitude and timing of peripheral waves. An increase in aortic stiffness alone only causes an increase aortic pulse pressure with little change in wave contour (64). Although this increases systolic and pulse pressure the increase is minor as compared to the increase caused by wave reflections (64). The later part of a recorded pressure wave is inversely related to the round trip travel time of the pressure wave and is generated by the reflected wave arriving during systole and adding to the forward traveling pressure wave and augmenting systolic and pulse pressure (64). The changes in systolic pressure that occur at the aorta cannot be determined by traditional measurements of blood pressure at the brachial artery. Therefore, the effects of arterial stiffening are most times ignored and not taken into account when therapeutic strategies are employed.

Endothelial Dysfunction and Coronary Artery Disease

Endothelial dysfunction, characterized by impaired vasodilation in response to stimuli such as acetylcholine or increased blood flow, has been documented in both conduit and resistance vessels in CAD and is associated with increased risk for future cardiovascular events (1,2,70). Additionally, endothelial dysfunction likely contributes to the increased arterial stiffness seen in CAD. Endothelial cells participate in control of vasodilation through the release of NO in response to a stimulus such as acetylcholine or increased blood flow (24,71). NO is produced enzymatically by endothelial NO-synthase (eNOS) from L-arginine and acts on smooth muscle through its second messenger cyclic guanosine monophosphate (cGMP) (24). The blood flow response to infused acetylcholine and increased blood flow is attenuated in CAD indicating a decreased stimulated release of NO (2,3). Several clinical studies have documented impaired

endothelium-dependent flow-mediated dilation (FMD) of the conduit arteries in CAD patients (8,21). It appears that the decreased vasodilatory response to increased blood flow in CAD is due in part to decreased production and/or availability of NO. Pretreatment with L-arginine, the substrate for NOS, improves endothelial dependent vasodialition in atherosclerotic rabbits and CAD patients (72,73). The functional consequence of reduced NO production in CAD is an inability to vasodilate in response to physiological stimuli, such as increased blood flow, leading to decreased tissue perfusion and an increased afterload leading to ischemia.

Arterial Stiffness and Coronary Artery Disease

As stated earlier, afterload is composed of peripheral resistance, arterial stiffness and the reflectance of pressure and flow. An increase of any of these components is especially detrimental in CAD. Central arterial stiffness is increased in coronary artery disease and myocardial infarction (74). An increase in arterial stiffness in CAD leads to increased myocardial oxygen demand and a decrease in coronary perfusion. As opposed to healthy individuals in whom maximal cardiac output is the main determinant of exercise capacity, CAD patients may be limited by myocardial ischemia determined by coronary perfusion and cardiac work. Aortic stiffening in animal studies using bandaging (75) or a stiff plastic tube bypass (76,77) results in increased pulse pressure and cardiac work and a reduction in coronary perfusion. Coronary perfusion was reduced particularly in the subendocardial region in the presence of circumflex stenosis and even more so with stimulated exercise through pacing (75). This evidence suggests that aortic stiffness increases myocardial oxygen demand and reduces myocardial perfusion particularly in the setting of CAD. In support of this, a recent investigation found that in 91 patients

with CAD, time to ischemia during treadmill testing was inversely correlated to arterial stiffness independent of gender, age, mean pressure, degree of disease, left ventricular mass and smoking status suggesting that arterial stiffness is a principal determinant of ischemic threshold in CAD (4).

The mechanism responsible for the increased arterial stiffness seen in CAD is likely due in part to endothelial dysfunction. Measurements of pulse wave velocity following NOS inhibition revealed that an intact NO generating endothelium is required to maintain normal arterial compliance (78). Furthermore, NOS inhibition has been shown to alter the peripheral pulse wave so that the return of the reflected wave occurs earlier (79). A second mechanism likely to contribute to increased arterial stiffness is the formation of advanced glycation end-products (AGE). AGE accumulate over the lifespan on long-lived proteins such as collagen and elastin forming crosslinks and stiffening the arteries (80).

Mechanism of Endothelial Dysfunction in CAD

The mechanism of endothelial dysfunction in CAD appears to be multi-factorial with possible mechanisms including: 1) decreased expression of eNOS or synthesis of NO, and 2) inactivation of NO by reactive oxygen species.

Expression of eNOS and Synthesis NO

Elevated concentrations of oxidized LDL, TNFα, and hypoxia can all lead to a down-regulation of eNOS expression by reducing eNOS mRNA half-life (81). A reduced expression of eNOS could be the result of increased levels of LDL in patients with CAD. Oxidized LDL downregulate the expression of eNOS (82). Additionally, autoantibodies against oxidized LDL are inversely related to acetylcholine-induced blood

flow in hypercholesterolemia (17). In healthy subjects, who presumably have normal eNOS expression, NO production is greater in exercise trained subjects (19). Therefore, it is hypothesized that exercise training will result in an improved production of NO in CAD regardless of pre-training eNOS expression.

Nitric Oxide and Reactive Oxygen Species

Enhanced biodegradation of NO by superoxide is a second possible mechanism for endothelial dysfunction in CAD. Nitric oxide and superoxide react in a diffusion limited reaction to form peroxynitrite which is a strong oxidant with only minimal vasodilator activity (57). A second possible mechanism for free radical induced endothelial dysfunctin is oxidation of tetrahydrobiopterin (BH_4) (83). BH_4 is an important cofactor in NO synthesis. Elevated markers of oxidative stress in CAD provide indirect evidence for increased production of oxygen free radicals (57,84). Both acute and chronic treatment with vitamin C normalized flow-dependent dilation in CAD patients supporting the hypothesis that oxygen free radicals are involved in the endothelial dysfunction seen in CAD (21). Bauersachs et al (85) demonstrated that pretreatment with exogenous SOD normalized vascular response to acetylcholine in aortas from rats with chronic myocardial infarction. This work demonstrates that inactivation of NO by superoxide is an important mechanism for endothelial dysfunction. The source of superoxide appears to be a smooth muscle NADH/NADPH dependent oxidase (85). Vascular smooth muscle cells generate superoxide in response to angiotensin II as a result of stimulation of an NADH/NADPH dependent oxidase (86). Long term ACE inhibitor ($\geq$ 12 weeks) treatment improves endothelial function in hypertensive patients (87). One likely mechanism for this is a relative increase in NO

availability subsequent to the inhibition of ANG II stimulated superoxide release (87). The same effect is likely to occur in coronary artery disease.

A reduction in antioxidant defenses may also contribute to oxidative stress in CAD. Extracellular SOD (EC-SOD) is the primary SOD isoform found in the vessel wall and plasma and is responsible for the majority of activity in plasma (88). Endothelium bound EC-SOD is the principal source of plasma EC-SOD, and levels are in equilibrium between these two phases (88). EC-SOD has been shown to be reduced in coronary arteries and plasma of CAD patients (22). Plasma EC-SOD levels were shown to be highly correlated with radial artery FMD (22). Previous cell and animal research has shown that SOD is up-regulated by shear stress (89) and exercise training (23). Therefore, we hypothesized that exercise training will result in an increase in plasma SOD activity in CAD.

Inflammation

It is now established that CAD is an inflammatory process and future cardiovascular events can be predicted based on selected inflammatory markers. The first work in this area involved prospective epidemiological studies of apparently healthy individuals. Increased vascular risk was found to be associated with increased basal levels of cytokines such as IL-6 and TNFα (12), cell adhesion molecules such as P selectin (90), and downstream acute phase reactants such as CRP and fibrinogen (12). C-reactive protein (CRP) is the most robust of these markers and interleukin-6 (IL-6) has also shown some promise as a predictor of cardiovascular events (12). The first data to provide evidence that CRP is predictive of cardiovascular events in persons with CAD came from the Cholesterol and Recurrent Events (CARE) trial (91). The CARE trial was

a secondary prevention trial that demonstrated that CRP levels correlated with a significantly increased risk of recurrent coronary events (91).

Because CRP has been shown to be an independent predictor of cardiovascular events in both healthy individuals and CAD patients one would hope that an intervention reducing CRP levels would reduce risk for future events as well. Aspirin therapy and statin therapy have both been shown to reduce both CRP and mortality (91,92). Although these medications have positive effects unto themselves their effectiveness may also be related to their anti-inflammatory actions as well (12). To date, however, there have been no investigations into whether a reduction in CRP affords a concomitant reduction in risk.

The Role of Exercise Training

Endothelial Dysfunction

There is an increasing body of evidence that exercise training has a beneficial effect on endothelial function. In a study of healthy young military recruits, a 10-week period of basic training consisting of daily 3-mile runs and upper body strength and endurance training resulted in an improvement in brachial artery FMD when compared to a control group of civilians (6). In older men, 3-months of home-based walking prevented age-related impairment of forearm endothelial function and restored microvascular function to that seen in younger adults (93). Similar findings have been found in diseased populations. In patients with congestive heart failure, 4 weeks of handgrip training improved radial artery FMD (94). N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOS), blocked this improvement providing evidence that the improvement in FMD with forearm exercise was due to enhanced nitric oxide release (94). In another study of heart failure patients, 6-months of stationary cycling resulted in an increase in endothelium-dependant femoral blood flow

(15). The same group of investigators recently reported a positive effect of 4-weeks of cycling on forearm radial artery responses, suggesting a systemic effect of endurance training on peripheral vascular reactivity (95). Endothelial function has also been shown to improve following 3-months of cycle training in a group of middle-aged men with polymetabolic syndrome (96). The investigators reported an improvement in brachial artery FMD without changes in body mass index, blood pressure, or lipids suggesting a direct beneficial effect of endurance exercise on systemic arterial function (96).

There have been only two prior studies that have examined the effect of exercise training on vascular reactivity in CAD patients. The first study examined only adaptation in the coronary circulation (3). In that study, inpatients performed high-intensity exercise for 60 minutes of cycling per day for 4 weeks. They reported improved coronary epicardial and resistance vessel response to acetylcholine and adenosine compared to outpatient controls who received similar pharmacological therapy but led a sedentary lifestyle (3). More recently the effects of 10-weeks of endurance exercise as part of a cardiac rehabilitation program, resulted in improved posterior tibilias FMD but only a positive trend in brachial artery FMD compared to a sedentary control group (8). The subjects underwent lower body endurance training which the authors suggest may not result in systemic improvements in endothelial function due to the non-significant improvement in brachial artery FMD (8). However, the brachial artery FMD improvement (6.4% to 8.3%), although not statistically significant, is likely clinically significant. Higashi et al (16) demonstrated improved brachial artery endothelial function in response to acetylcholine following 6 weeks of walking in a group of hypertensive subjects. These findings are consistent with those of Linke and colleagues

(95) that demonstrated that lower body exercise training provides systemic improvements in endothelial function. Based on these results we hypothesize that exercise training would result in improved FMD of the brachial artery in patients with CAD.

Arterial Stiffness

Exercise training appears to be associated with improved arterial stiffness and compliance in both animals (97) and healthy humans (98). When the effects of advancing age are controlled for, arterial stiffness is less in persons with a higher VO_{2max} (99). Exercise training in spontaneously hypertensive rats also results in improved aortic compliance (100). Tanaka and coworkers (101) reported increased measures of central arterial compliance in middle-aged men following 3 months of aerobic walking. Exercise training induced alterations in aortic stiffness or compliance would be of great benefit in CAD. Vasodilator therapy using nitrates or ACE inhibitors is effective in reducing wave reflections (59) and it is possible that exercise training may have the same effect. The decreased afterload could possibly result in increased exercise tolerance and a reduction in angina symptoms. The proposed study would be the first to study the effects of exercise training on arterial stiffness in CAD.

Nitric Oxide Release

To date there have been no reports of the effect of exercise training on the release of nitric oxide in CAD. Exercise training has the potential to alter the release of nitric oxide based on research in animals and healthy subjects. Exercise is accompanied with increased blood flow to meet the metabolic demands of the exercising muscles. Animal studies have shown an increase in endothelial nitric oxide synthase (eNOS) mRNA (44) and eNOS protein (18) expression in the aortic wall following exercise training. Five days of treadmill running in rats resulted in improved vasodilatory response to

acetylcholine (102). In humans, resting plasma levels of nitrates, a measure of NO production, are greater in exercise trained versus untrained healthy volunteers (19). As a group, these studies indicate that exercise training has a beneficial effect on NO production but the effect of exercise training in CAD is yet unknown.

NO and Reactive Oxygen Species

The inactivation of NO by superoxide is yet another mechanism of endothelial dysfunction in CAD. Exercise training stimulates an up-regulation of SOD activity in oxidative skeletal muscle of rats (103) and VO_{2max} is linearly related to vastus lateralis SOD activity in humans (104). Twelve weeks of treadmill exercise in pigs resulted in increases in coronary arteriole Cu/Zn SOD, Cu/Zn SOD activity and Cu/Zn SOD mRNA but no changes in Mn SOD or catalase (105). Extracellular SOD (EC-SOD) is the primary SOD isoform found in the vessel wall and plasma and is responsible for the majority of activity in plasma (88). Endothelium bound EC-SOD is the primary source of plasma EC-SOD, and levels are in equilibrium between these two phases (88). EC-SOD has been shown to be reduced in coronary arteries and plasma of CAD patients (22). Plasma EC-SOD levels were shown to be highly correlated with radial artery FMD (22). Previous cell and animal research has shown that SOD is up-regulated by shear stress (89) and exercise training (23). This increase in SOD appears to be NO dependent. Three weeks of treadmill training in wild type mice increased aortic eNOS protein expression 3.2 fold and EC-SOD protein expression 2.8 fold whereas as aortic Cu/Zn did not change with training (23). In contrast to these findings, exercise training had no effect on EC-SOD protein levels in eNOS -/- mice (23). The authors speculate that this represented an important feed forward mechanism of NO-induced increases in EC-SOD

expression whereby NO released by the endothelium enhanced its own biological effects by reducing superoxide.

The effects of exercise training on oxidative stress and antioxidant capacity have yet to be investigated in CAD. However, we hypothesize that exercise training will lead to an increase in plasma SOD activity.

Inflammation

It is now established that CAD is an inflammatory process and future cardiovascular events can be predicted based on selected inflammatory markers. C-reactive protein (CRP) is the most robust of these markers and interleukin-6 (IL-6) has also shown some promise as a predictor of cardiovascular events (12). We found that CRP levels are reduced (33%) in CAD patients following 12 weeks of exercise training. However, IL-6 was not significantly reduced. The findings of the present study are consistent with previous findings from our laboratory. Previously, we had studied 11 CAD patients participating in standard cardiac rehabilitation. We found a significant decrease in CRP and a trend ($p=0.1$) toward a reduction in IL-6. Prior to studies conducted in our lab, there had only been one cross sectional study investigating the relationship between exercise and CRP (13). In this study physical activity was associated with lower CRP levels (13). Because CRP has been shown to be an independent predictor of cardiovascular events in both healthy individuals and CAD patients one would hope that an intervention reducing CRP levels would reduce risk for future events as well. Aspirin therapy and statin therapy have both been shown to reduce both CRP and mortality (91,92). Although these medications have positive effects unto themselves their effectiveness may also be related to their anti-inflammatory actions as

well (12). To date, however, there have been no investigations into whether a reduction in CRP affords a concomitant reduction in risk.

Summary

In summary, the mechanism of endothelial dysfunction in CAD appears to be multi factorial. Exercise training has the potential to improve endothelial function and arterial stiffness in a variety of ways. Alterations in endothelial function and arterial stiffness may help explain the beneficial effects of exercise training in CAD. The experiments presented in this proposal are novel and will further the understanding of the pathophysiology of CAD and clarify the role of exercise training in the treatment of CAD. Exercise training is an inexpensive adjunct treatment for CAD and has been shown to improve quality of life. For that reason, future research in the area of exercise training in CAD is extremely important.

CHAPTER 3
METHODS

The experiments in this proposal were designed to investigate the effects of 12 weeks of cardiac rehabilitation (standard of care in the USA) on endothelial function in coronary artery disease (CAD) patients. A total of 20 patients were recruited and studied prospectively. Ten patients were enrolled in a standard 12-week exercise training program at the Shands Hospital at AGH Cardiac Rehabilitation Program and 10 patients without access to cardiac rehabilitation will serve as non-exercise controls. CAD patients were studied before and after 12 weeks of exercise training to determine the effects of exercise training on endothelial function, arterial stiffness, NO production, oxidative stress, and antioxidant defenses. The study was approved by the University of Florida Health Science Center Institutional Review Board.

Subjects

All CAD patients were recruited from the Shands at AGH Cardiac Rehabilitation Program. All patients were diagnosed with CAD as documented by myocardial infarction and/or catheterization. The selection of subjects was not be based on gender or racial/ethnic status.

Inclusion Criteria

1. Age 18 to 75
2. Documented CAD
3. Optimal medical therapy (aspirin, ACE-inhibitors, β -blockers, statins)

Exclusion Criteria

1. Acute unstable myocardial ischemia/angina
2. Myocardial infarction within 6 weeks
3. History or clinical evidence of moderate or severe hypertension
4. Diabetes mellitus
5. Other non-cardiac diseases that would interfere with completion of the study (e.g. thyrotoxicosis, anemia, lung disease, renal failure)
6. Patients with cardiac pacemakers
7. Orthopedic problems that would limit exercise
8. Claudication or peripheral vascular disease
9. Idiopathic cardiomyopathy

Group Assignments

Twenty patients were assigned to either 12 weeks of standard cardiac rehabilitation or a 12 week control period. Because of the benefits of exercise in this population we feel it would be unethical to withhold exercise from patients who would ordinarily have access to cardiac rehabilitation. Thus, we decided to use a cohort of CAD patients who did not have access to cardiac rehabilitation as our control group. This was made possible by the fact that some patients travel to Shands at AGH from outlying communities without cardiac rehabilitation programs and not all insurance programs reimburse for cardiac rehabilitation. We administered a physical activity questionnaire at the conclusion of the study to insure that no control subjects participated in a regular exercise program.

Exercise Training

Exercise training took place in the Shands Hospital at AGH Cardiac Rehabilitation Program, Gainesville, FL. CAD patients referred to Shands at AGH who were candidates for cardiac rehabilitation and lived in Gainesville, FL area were asked to participate in the study. Training sessions were be under direct supervision by an exercise specialist and a registered nurse and overseen by a physician. Exercise consisted of treadmill walking, stationary cycling and upper body ergometry 3 times per week for 12 weeks. Twelve weeks duration was chosen to match current insurance reimbursement guidelines. Therefore, statements can be made with reference to care patients are actually receiving. Exercise intensity began at 40-50% of maximum heart rate reserve or as symptoms allowed and gradually progressed to 70-85% of maximum heart rate reserve. The duration of each exercise session began at 15-20 minutes or as tolerated and progressed to 40-50 minutes of sustained walking and cycling. A log of HR, BP, duration, rating of perceived exertion, treadmill speed and grade, and cycle workload was be kept for each exercise training session.

Specific Measurements

Subjects were asked to make a total of 4 visits during the study for testing. Details of the study protocol are outlined in Figure 3-1.

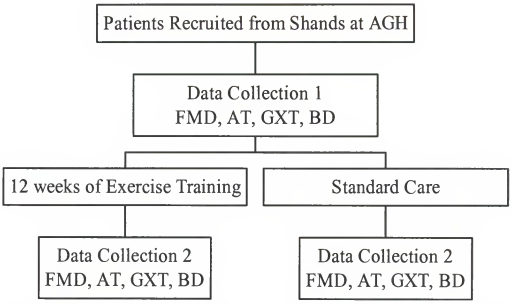

Figure 3-1. Research protocol. FMD = flow mediated dilation, AT = applanation tonometry, GXT = graded exercise test, BD = blood draw.

Endothelial Function: Brachial Artery Reactivity Testing

Brachial artery reactivity testing was performed using high resolution ultrasound (HDI 3000, ATL, Inc). Vascular reactivity measurements were be made at least 4 hours after the subject's last meal and the subject was asked to eat a low fat diet on the day of testing (106). Brachial artery vasodilatory responses to hyperemia were measured in the right brachial artery. A 10 MHz linear phased array ultrasound transducer was used to image the brachial artery longitudinally. After recording baseline measurements of end diastolic diameter and blood flow velocity a cuff was inflated proximally to 200 mmHg for 5 minutes. Recent research has shown that this cuff position results in the greatest increase in blood flow and vasodilation (107). After release of the cuff brachial artery blood flow velocity and diameter were measured during the first minute of reactive hyperemia. This flow-mediated dilation (FMD) was used as a measure of endothelial dependent function and expressed as a percent increase from baseline. It has been rshown that the vasodilatory response to NO donors (endothelial independent dilation) is normal

in stable CAD patients (108,109). Therefore, endothelial independent function with the use of nitroglycerin was not tested in this group of patients to reduce the risk of hypotensive episodes. Brachial artery reactivity testing was repeated following 12 weeks of exercise or a 12-week control period. Measurements took place on the 11th floor of Shands Hospital at the University of Florida in the Vascular Laboratory.

Arterial Stiffness

Measurements of arterial stiffness were made at the same visit as measures of endothelial function. Subjects were supine in a quiet room for at least 10 minutes before measurements were obtained and remained supine during testing. Arterial blood pressure was determined in duplicate from the right arm using an automated blood pressure device (Omron HEM 735). High-fidelity pressure waveforms were recorded non-invasively by applanation tonometry in the radial artery using a pencil-type Millar Micro-tip pressure transducer. Optimal recording of the pressure wave was obtained when the hold-down force of the transducer on the artery was such that the resulting representative waveform had a stable baseline for at least 10 beats. The amplitude of central systolic arterial wave reflection was estimated by the augmentation index (AIx), which was obtained from the configuration of the generated aortic pressure waveform. In adults above approximately 20 years of age and average height, a distinct inflection point (Pi) occurs in systole either before or after peak pressure (Ps) (59). AI is calculated as (Ps-Pi)/(Ps-Pd). Since AI is a dimensionless ratio, the units of calibration do not affect its value. AI is a highly reproducible parameter, simple to measure and an indicator of arterial stiffness. The aortic pressure was synthesized using the Sphygmocor pulse wave analysis system (SCOR Px, Atcor Medical). This system uses a generalized transfer function to estimate the aortic pressure wave from the radial pressure wave. Synthesized aortic pressure

waves have been shown to be almost identical to invasively recorded central pressure waves (110). This system has also shown good reproducibility (111).

The measured arterial pressure wave is the sum of a forward traveling wave and a reflected wave superimposed upon the mean blood pressure. These two waves travel in opposite directions along the artery at the same velocity. The round-trip travel time (Δt) of the forward wave from the heart to the major reflection site and back (reflected) is measured from the foot of the forward traveling pressure wave to Pi (i.e. the upstroke of the reflected wave). The one-way travel time of the reflected pressure wave from the periphery to the heart is measured as $\Delta t_p/2$. This measurement is directly related to reflection site distance and inversely related to arterial stiffness or pulse wave velocity over this distance (59).

Graded Exercise Test

All patients enrolled in the study will underwent a symptom limited graded exercise test (GXT) to determine peak oxygen consumption (VO_{2peak}) before and after 12 weeks of exercise training or the control period. Testing will took place in the Metabolic Laboratory in the Center for Exercise Science, University of Florida, Gainesville, FL. The lab is equipped with a motorized treadmill and 12 lead electrocardiogram recorder (Quinton) and metabolic cart (ParvoMedics). A 20-gauge catheter was be placed into the anticubital vein prior to testing. After a 30 minute rest period a 20 ml blood sample was drawn for the analysis plasma levels of NO, oxidative stress, and inflammation. A walking symptom limited graded exercise test was performed using a modified Naughton protocol which begins at 2 mph, 0% grade and progressed by 2% grade every 2 minutes.

The test provided VO_{2peak} and peak heart rate used in exercise prescription. All tests were under the supervision Richard Schofield, M.D.

Criteria for Termination of Exercise Tests:

1. Fatigue
2. Light-headedness, confusion, ataxia, cyanosis, dyspnea, or nausea
3. Onset of angina with exercise
4. Symptomatic supraventricular tachycardia
5. 3 mm horizontal or downsloping ST depression
6. Ventricular tachycardia (4 or more consecutive PVC's)
7. Exercise induced left bundle branch block
8. Onset of second or third degree A-V block
9. R on T PVC
10. Frequent multifocal PVC's (30% of complexes)
11. Excessive hypotension (greater than 20 mm Hg drop in systolic BP)
12. Excessive hypertension (systolic BP > 220 mm Hg; diastolic BP > 110 mm Hg)
13. Inappropriate bradycardia (HR drop > 10 bts/min)

Blood Collection

Blood was collected in tubes containing EDTA and immediately underwent centrifugation at 3,000 rpm for approximately 15 minutes. Plasma was aliquoted into several tubes for storage. Plasma that was to be used for measurement of oxidative stress was stored with diethylenetriamine pentaacetic acid (DTPA) and butylated hydroxytoluene (BHT) at a concentration of 0.01mM. All plasma samples were stored at –80°C for analysis at the end of the study.

Biochemical Analyses

Nitrite/Nitrate Measurement

Because NO is rapidly converted to nitrate and nitrite measurement of these metabolites was used to estimate NO production. Plasma nitrate/nitrite was measured using a kit (Cayman Chemical). First any plasma nitrate was converted to nitrite using nitrate reductase. Spectrophotometric analysis of total nitrite was then performed using

Greiss reagent. Subjects were be asked to follow a low nitrate diet for 24 hours prior to testing.

8-isoprostane-$F_{2\alpha}$

Oxidative stress was assessed by measuring plasma levels of 8-isoprostane-$F_{2\alpha}$. A competitive ELISA (Cayman Chemical) was performed to assess plasma levels of 8-isoprostane-$F_{2\alpha}$. 8-isoprostane-$F_{2\alpha}$ in the plasma sample competes for binding with 8-isoprostane-$F_{2\alpha}$ covalently attached to alkaline phosphatase. The plate is then incubated with p-nitrphenyl phosphate and the reaction stopped the addition of and acid. The plate is read at 405 nm and the absorbance is inversely proportional to 8-isoprostane-$F_{2\alpha}$ in the samples.

SOD, GPX and Total Antioxidant Status

Total plasma SOD activity was assayed using a modified nitrite technique (112). Superoxide generated by hypoxanthine and xanthine oxidase forms nitrite in the presence of hydroxylamine. Nitrite is then measured at 550 nm using a coloring reagent. One unit of SOD activity is defined as the amount of SOD required for a 50% decrease in nitrite formation. Glutathione peroxidase activity was assayed utilizing the coupled reaction of GPX and glutathione reductase (113). The rate at which NADPH is oxidized to recycle glutathione is measured using spectrophotometer at 340 nm. Total antioxidant capacity was measured using a kit provided by Calbiochem. The assay relies on the ability of antioxidants in the plasma sample to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenz-thiazoline sulphonate) by metmyoglobin, a peroxidase after the addition of hydrogen peroxide. Oxidized ABTS was then be read using a spectrophotometer at 600 nm.

C-reactive Protein, Interleukin-6

C-reactive protein was measured using a sandwich ELISA (Alpha Diagnostic). The microplate is coated with one antibody to CRP and a second antibody conjugated with horseradish peroxidase is added following the sample. The product of HRP can be read at 450 nm. Similarly, interleukin-6 was also measured using a sandwich ELISA (Cayman Chemical). In this instance the second antibody was conjugated with acetylcholinesterase. It's product can be read at 405 nm.

Statistical Considerations

Analysis of variance (ANOVA) with repeated measures was used to analyze the measures of endothelial function, arterial stiffness, plasma markers of vascular function and oxidative stress in CAD patients before and after 12 weeks of cardiac rehabilitation or the control period. When a significant group by time interaction was observed, within-group post hoc comparisons between time points and between –group post hoc comparisons at each time point was performed using Tukey's post hoc analyses. All statistical analyses were performed using the NCSS statistical program. An alpha level of $p<0.05$ was required for statistical significance. A power analysis was performed to estimate the statistical power related to testing the following hypothesis in 20 patients: 12 weeks of exercise training will result in greater flow-mediated dilation of the brachial artery when compared to a 12 week control period. The statistical power related to testing the hypothesis that flow-mediated dilation of the brachial artery is greater in exercise trained CAD patients (compared to control CAD group) is 0.92 for a 2-tailed test when the group means were conjectured to be 8% and 14% for control and exercise trained patients respectively; the standard deviation is assumed to be 0.1 mm, the total sample size 20 patients, and the alpha level set at 0.05.

Ethical Aspects

Confidentiality

No personal identifiers will be used in any publication or presentation resulting from this study. Information will be made available to the patient's physician following signed release.

Risks to Subjects

The measurements of endothelial function and arterial stiffness are non-invasive and presented minimal risk to patients. There was minimal risk associated with the venous catheter placement used in this study. The risks were possible bruising and swelling around the stick site, rarely an infection, and uncommonly faintness from the procedure. Universal precautions mandated by the Center for Disease Control and OSHA were used in the handling of human blood and urine.

Graded exercise testing carries a minimal risk of cardiac events (1/10,000) and patients were made aware of this risk prior to testing. However, graded exercise testing is standard procedure for evaluation of functional capacity and the effectiveness of both exercise and pharmacological interventions. All tests were supervised by a physician.

The risk to subjects during exercise training was minimal and training sessions were under the direct supervision of an exercise specialist and a registered nurse and overseen by a physician. Endurance exercise training has been shown to be both safe and effective in treating CAD patients.

CHAPTER 4
RESULTS

Throughout the results section C will refer to the control group and ET to exercise trained group. BSLN will refer to measurements performed at baseline before the initiation of training or the control period and 12 Weeks refers to measurements performed at the end of the 12 weeks training period or control period.

Subject Characteristics

The characteristics for the participants in this study are presented in table 4-1. The groups did not differ with respect to any of the variables listed.

Table 4-1. Baseline Characteristics

	Control (n=10)	Exercise (n=10)
Age (yrs)	57.6±8.1	63.4±8.6
Gender	9 M, 1 F	9 M, 1 F
Height (cm)	174.8±8.2	175.5±7.8
Weight (kg)	87.2±25.7	91.8±20.9
Body mass index (kg/m2)	28±7.2	30±5.5
Coronary bypass (n)	6	5
Percutaneous coronary intervention (n)	4	5
Myocardial infarction (n)	6	4
ACE inhibitor therapy (n)	3	2
Lipid lowering therapy (n)	2	4
Nitrate therapy (n)	2	4

ACE = angiotensin converting enzyme.

Endothelial Function

Brachial artery flow mediated dilation (FMD) is presented in Table 4-2 and Figure 4-1. As shown in Figure 4-1 the ET group significantly increased FMD following

12 weeks of exercise training (7.2% v. 11.2%, $p<0.05$) but there was no change in FMD in the C group after the control period.

Table 4-2. Brachial artery reactivity testing

	CONTROL		Exercise Trained	
	BSLN	2 WEEKS	BSLN	12 Weeks
Baseline diameter (mm)	4.07±0.66	3.9±0.63	4.25±0.73	4.14±0.92
Absolute dilation (mm)	0.35±0.11	0.32±0.09	0.33±0.11	0.51±0.17
Brachial FMD (%)	8.0±2.4	8.4±2.3	7.2±2.1	11.2±3.3*

Values are mean ± SE. *$p<0.05$ vs. BSLN. FMD = flow mediated dilation.

Pulse Wave Analysis

Results from pulse wave analysis of the radial artery are presented in Table 4-3 and Figures 4-2 and 4-3. Twelve weeks of endurance exercise training did not alter heart rate, central or peripheral systolic, diastolic or pulse pressures but did result in a decrease in augmentation index (AIx) (30.4% v. 26.2%) and an increase in the delay of the reflected wave (Δt) (136.2 v 144.4) in the ET group. There were no changes in any of these parameters in the C group during the 12-week control period.

Table 4-3. Pulse wave analysis

	Control		Exercise Trained	
	BSLN	12 Weeks	BSLN	12 Weeks
HR (bt/min)	68.8±5.1	70.2±7.6	61.1±7.5†	60.0±6.2†
PSBP (mmHg)	127.3±6.0	126.8±3.6	130.4±7.3	126.2±2.7
PDBP (mmHg)	75.8±11.3	74.5±9.6	78.0±6.9	79.0±3.9
PPP (mmHg)	51.5±11.9	52.3±10.5	52.4±7.4	47.2±4.4
CSBP (mmHg)	116.8±5.8	115.0±4.2	120.4±9.0	116.4±3.9
CDBP (mmHg)	76.5±11.1	75.3±9.9	81.0±6.2	79.0±5.1
CPP (mmHg)	40.3±9.9	39.8±10.5	39.4±6.2	37.4±5.2
AIx (%)	28.1±2.6	27.2±3.8	29.9±5.0	26.2±2.7*
Δt (ms)	131.8±3.71	132.6±3.64	136.2±1.21	144.4±2.44*

Values are mean ± SE. *$p<0.05$ vs. BSLN. $^{\dagger}p<0.05$ vs. Control. HR = heart rate, PSBP = peripheral systolic blood pressure, PDBP = peripheral diastolic blood pressure, PPP = peripheral pulse pressure, CSBP = central systolic blood pressure, CDBP = central diastolic blood pressure, CPP = central pulse pressure, AIx = augmentation index, Δt = round trip travel time.

Vasoactive Balance

Baseline plasma levels of nitrate/nitrite (NOx) were similar in ET and C. Exercise training resulted in significantly greater NOx levels in ET (Table 4-2, Figure 4-3) but no changes were observed in the C group.

Table 4-4. Plasma nitrate/nitrite levels

	Control		Exercise Trained	
	BSLN	12 WEEKS	BSLN	12 WEEKS
NOx (μmol/L)	29.13±6.22	30.16±5.64	28.62±8.03	34.70±8.67*

Values are mean ± SE. *p<0.05 vs. BSLN. NOx = nitrate/nitrite.

Oxidative Stress and Antioxidants

Results from measures of plasma oxidative stress and antioxidants are presented in Table 4-5. Baseline plasma levels of 8-isoprostane-$F_{2\alpha}$ (8-ISO) were similar in C and ET. Antioxidant defenses (superoxide dismutase (SOD), glutathione peroxidase (GPx) and total antioxidant status (TAS)) were also similar between both groups at baseline. Exercise training resulted in a significant reduction in 8-ISO and an increase in SOD activity in the ET group but no change was seen in the C group (Figures 4-8 & 4-9). Plasma GPx and TAS did not change in either group over the course of the 12 weeks (Figures 4-10 & 4-11)

Table 4-5. Markers of oxidative stress and plasma antioxidants

	Control		Exercise Trained	
	BSLN	12 Weeks	BSLN	12 Weeks
8-ISO (pg/ml)	455.9±60.7	482.0±80.5	490.5±140.5	406.7±109.8*
SOD (U/ml)	1.50±0.19	1.46±0.09	1.49±0.15	1.64±0.31*
GPx (μmol/ml)	0.149±0.014	0.147±0.015	0.140±0.014	0.144±0.010
TAS (mM)	0.836±0.19	0.814±0.24	0.867±0.29	0.928±0.19

Values are mean ± SE. *p<0.05 vs. BSLN. 8-ISO = 8-isoprostane-$F_{2\alpha}$, SOD = superoxide dismutase, GPX = glutathione peroxidase, T ANT = total antioxidant status.

Inflammation

Results from measures of inflammation are presented in Table 4-6. Baseline plasma levels of CRP and IL-6 were similar in ET and C. Exercise training resulted in significant reduction of CRP levels in ET only and there was no change in IL-6 in the ET group or C group over time (Figure 4-12 & 4-13).

Table 4-6. Markers of inflammation

	CONTROL		Exercise Trained	
	BSLN	12 Weeks	BSLN	12 Weeks
CRP (mg/dl)	0.296±0.17	0.298±0.19	0.276±0.19	0.180±0.08*
IL-6 (pg/ml)	5.43±2.25	5.39±3.32	5.64±2.21	4.91±3.13

Values are mean ± SE. *p<0.05 vs. BSLN. CRP = C-reactive protein, IL-6 = interleukin-6.

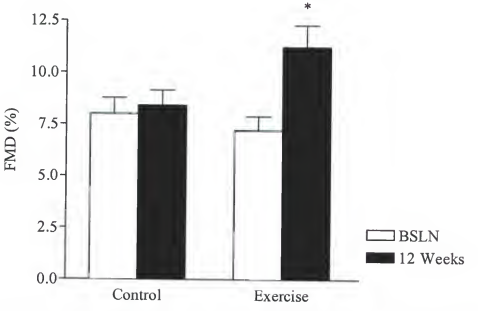

Figure 4-1. Flow mediated dilation. Values are mean ± SE. *p<0.05 vs. BSLN.

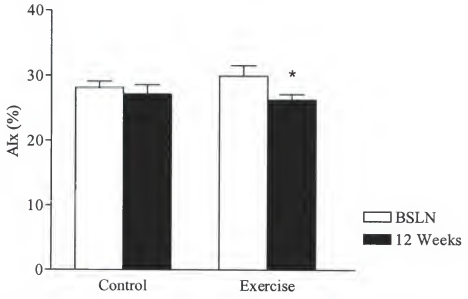

Figure 4-2. Augmentation index (AIx). Values are mean ± SE. *p<0.05 vs. BSLN.

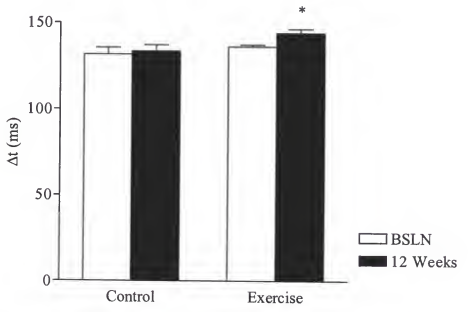

Figure 4-3. Delay of reflected wave. Values are mean ± SE. *p<0.05 vs. BSLN.

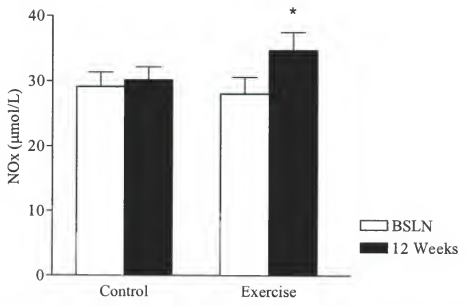

Figure 4-4. Plasma nitrate/nitrite. Values are mean ± SE. *p<0.05 vs. BSLN.

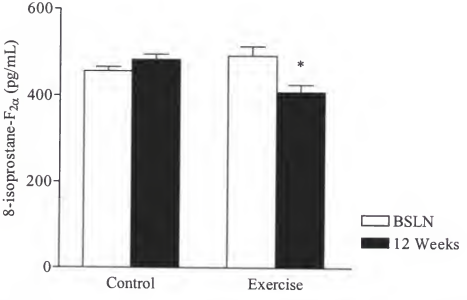

Figure 4-8. Plasma 8-isoprostane-$F_{2\alpha}$. Values are mean ± SE. *p<0.05 vs. BSLN.

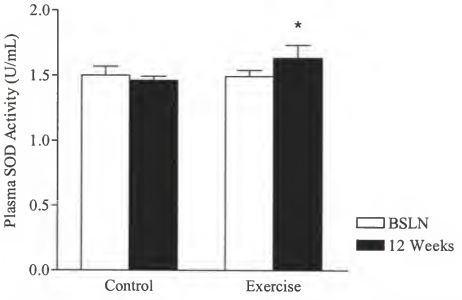

Figure 4-9. Total superoxide dismutase activity. Values are mean ± SE. *p<0.05 vs. BSLN.

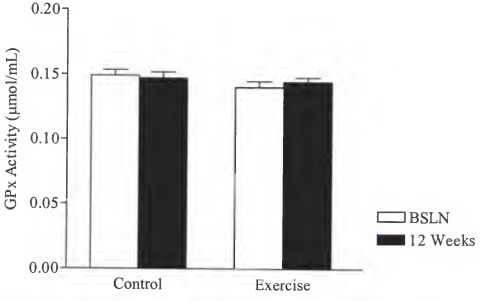

Figure 4-10. Plasma glutathione peroxidase activity. Values are mean ± SE.

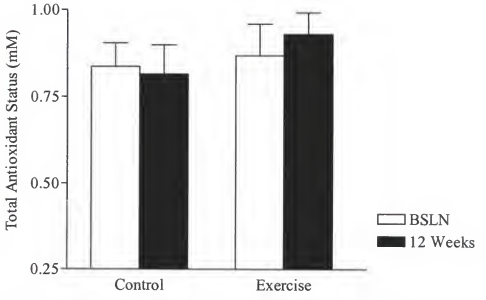

Figure 4-11. Plasma total antioxidant status. Values are mean ± SE.

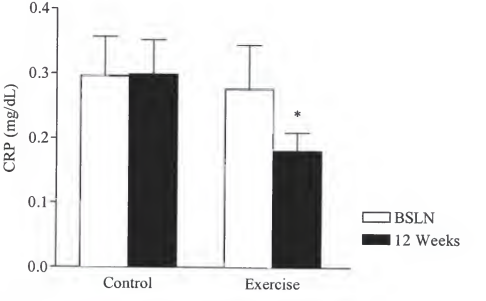

Figure 4-12. Plasma C-reactive protein. Values are mean ± SE. *p<0.05 vs. BSLN.

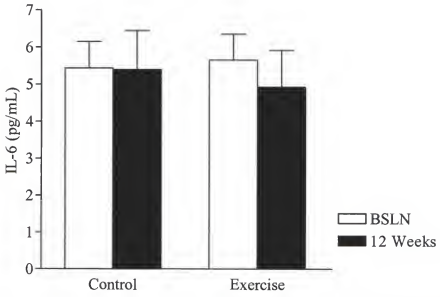

Figure 4-13. Plasma interleukin-6. Values are mean ± SE. *p<0.05 vs. BSLN.

CHAPTER 5
DISCUSSION

This is the first prospective study to evaluate the effects of endurance exercise as prescribed in cardiac rehabilitation (the standard of care in the United States) on vascular function, plasma oxidative stress and inflammation in patients with documented coronary artery disease (CAD). The major findings of the present study are that a supervised 12-week endurance exercise training program improves endothelial function and arterial stiffness and reduces oxidative stress and inflammation in patients with documented CAD. The relevance of these findings to the current literature is outlined below.

Endothelial Function

The current study demonstrated that 12-weeks of endurance exercise training in CAD patients leads to an improvement in endothelial function as measured by brachial artery flow mediated dilation (FMD)(7.2% at BSLN v. 11.2% at 12 Weeks). The results of the present study are consistent with previous studies that have demonstrated improved endothelium-dependent arterial vasodilation after exercise training. In a study of healthy young military recruits, a 10-week period of basic training consisting of daily 3-mile runs and upper body strength and endurance training resulted in an improvement in brachial artery FMD when compared to a control group of civilians (6). In older men, 3-months of home-based walking prevented age-related impairment of forearm endothelial function and restored microvascular function to that seen in younger adults (93). Similar findings have been found in diseased populations. In patients with congestive heart failure, 4 weeks of handgrip training improved radial artery FMD (94). N^G-monomethyl-L-

arginine (L-NMMA), an inhibitor of nitric oxide synthase (NOS), blocked this improvement providing evidence that the improvement in FMD with forearm exercise was due to enhanced nitric oxide release (94). In another study of heart failure patients, 6-months of stationary cycling resulted in an increase in endothelium-dependant femoral blood flow (15). The same group of investigators recently reported a positive effect of 4-weeks of cycling on forearm radial artery responses, suggesting a systemic effect of endurance training on peripheral vascular reactivity (95). Endothelial function has also been shown to improve following 3-months of cycle training in a group of middle-aged men with polymetabolic syndrome (96). The investigators reported an improvement in brachial artery FMD without changes in body mass index, blood pressure, or lipids suggesting a direct beneficial effect of endurance exercise on systemic arterial function (96).

There have been only two prior studies that have examined the effect of exercise training on vascular reactivity in CAD patients. The first study examined only adaptation in the coronary circulation (3). In that study, inpatients performed high-intensity exercise for 60 minutes of cycling per day for 4 weeks. They reported improved coronary epicardial and resistance vessel response to acetylcholine and adenosine compared to outpatient controls who received similar pharmacological therapy but led a sedentary lifestyle (3). More recently the effects of 10-weeks of endurance exercise as part of a cardiac rehabilitation program, resulted in improved posterior tibilias FMD but only a positive trend in brachial artery FMD compared to a sedentary control group (8). The subjects underwent lower body endurance training which the authors suggest may not result in systemic improvements in endothelial function due to the non-significant

improvement in brachial artery FMD (8). However, the brachial artery FMD improvement (6.4% to 8.3%), although not statistically significant, is likely clinically significant. The significant improvement in brachial artery FMD found in the present study suggests that lower body endurance exercise leads to a systemic improvement in endothelial function. These results are consistent with previous investigations using lower body endurance exercise that demonstrated improvements in brachial artery function in patients with heart failure or hypertension (16,95). The present study, in addition to the work by Gokce and colleagues (8), demonstrates that endurance exercise training as part of a cardiac rehabilitation program, the standard of care in the U.S., has a beneficial effect on the vasculature by improving endothelial function.

Pulse Wave Analysis

Pulse wave analysis in the present study demonstrated that 12-weeks of endurance training in CAD patients resulted in a reduction in arterial stiffness as evidenced by a reduction in augmentation index (AIx) (29.9% at BSLN v. 26.2% at 12 Weeks) and an increase in delay of the reflected wave (Δt) (136.2 ms at BSLN v. 144.4 ms at 12 Weeks). Although there has not been a great deal of research done in the area of exercise and arterial stiffness, there is some evidence that supports the findings of the present study. In healthy individuals, higher aerobic fitness levels have been shown to be associated with lower central arterial stiffness as measured by pulse wave analysis (9), pulse wave velocity (10) and MRI (11) suggesting that large artery arterial stiffness may be influenced by exercise capacity. Additionally, systemic arterial compliance correlated positively, whereas β-index, a measure of arterial stiffness of the aortic arch, was inversely correlated with maximal oxygen consumption ($VO_{2\,max}$)(4). Also, time to

exhaustion on a treadmill was positively correlated with arterial compliance and negatively correlated with rate-pressure product (114).

As opposed to healthy individuals in whom maximal cardiac output is the main determinant of exercise capacity, CAD patients may be limited by myocardial ischemia determined by coronary perfusion and cardiac work. Aortic stiffening in animal studies using bandaging (75) or a stiff plastic tube bypass (76,77) results in increased pulse pressure and cardiac work and a reduction in coronary perfusion. Coronary perfusion was reduced particularly in the subendocardial region in the presence of circumflex stenosis and even more so with stimulated exercise through pacing (75). This evidence suggests that aortic stiffness increases myocardial oxygen demand and reduces myocardial perfusion particularly in the setting of CAD. In support of this, a recent investigation found that in 91 patients with CAD, time to ischemia during treadmill testing was inversely correlated to arterial stiffness independent of gender, age, mean pressure, degree of disease, left ventricular mass and smoking status suggesting that arterial stiffness is a principal determinant of ischemic threshold in CAD (4).

To date there have been only two prospective studies examining the effects of endurance exercise training on elastic properties of the arterial system. Thirty minute of cycling at 65% of $VO_{2\,max}$ 3 times/week for 4 weeks resulted in a 30% improvement in arterial compliance in healthy individuals (98). A cross sectional study found that arterial compliance was higher in endurance trained middle age and older men compared to sedentary and recreationally active men of the same age (101). The same study reported that 3-months of aerobic exercise in 20 of the sedentary middle-age and older men

resulted in an increase in arterial compliance that was similar to the endurance trained group of men (101).

The present study is the first to investigate the effects of endurance training on arterial properties using pulse wave analysis in addition to being the first to investigate the exercise effect in CAD patients. Our results suggest that in patients with CAD, endurance exercise training improves systemic arterial stiffness. The improvements in arterial stiffness are likely due to improvements in endothelial function of the muscular arteries. There have been several reports in both animal models and humans that provide evidence that nitric oxide (NO) production by the endothelium plays a critical role in modulating arterial compliance (78,79,115)). Therefore, the improvements in endothelial function seen in the present study may account for the improvements seen in arterial stiffness.

Biochemical Analyses

Nitrate/Nitrite

Twelve weeks of endurance exercise in the present study resulted in an increase (21%) in resting plasma nitrate/nitrite (NOx) levels. Our finding is consistent with a previous cross-sectional study that reported higher resting plasma nitrate levels in endurance-trained individuals compared to sedentary controls (19). Additionally, 8 weeks of aerobic training resulted in increased NOx levels in healthy young individuals (Maeda 2001). Animal studies have shown an increase in endothelial nitric oxide synthase (eNOS) mRNA (44) and eNOS protein (18) expression in the aortic wall following exercise training. The results of the present study suggest that plasma NOx levels in patients with CAD are improved following endurance exercise training and the possible mechanism is an increase in eNOS expression.

Oxidative Stress and Antioxidants

There is evidence suggesting that increased vascular production of superoxide contributes to impaired NO action in patients with atherosclerosis (5). Another feature of atherosclerosis is vascular accumulation of oxidized LDL. Oxidized LDL is toxic and inhibits NO release from endothelial cells and may also inactivate NO directly (116). We have found that 12 weeks of exercise training in CAD results in a reduction in oxidative stress (17%) and an increase in total plasma SOD activity (10%). Extracellular SOD (EC-SOD) is the primary SOD isoform found in the vessel wall and plasma and is responsible for the majority of activity in plasma (88). Endothelium bound EC-SOD is the primary source of plasma EC-SOD, and levels are in equilibrium between these two phases (88). EC-SOD has been shown to be reduced in coronary arteries and plasma of CAD patients (22). Plasma EC-SOD levels were shown to be highly correlated with radial artery FMD (22). Previous cell and animal research has shown that SOD is up-regulated by shear stress (89) and exercise training (23). This increase in SOD appears to be NO dependent. Three weeks of treadmill training in wild type mice increased aortic eNOS protein expression 3.2 fold and EC-SOD protein expression 2.8 fold whereas as aortic Cu/Zn did not change with training (23). In contrast to these findings, exercise training had no effect on EC-SOD protein levels in eNOS -/- mice (23). The authors speculate that this represented an important feed forward mechanism of NO-induced increases in EC-SOD expression whereby NO released by the endothelium enhanced its own biological effects by reducing superoxide. Although we did not specifically measure EC-SOD we did see an increase in plasma total SOD activity. We speculate that this increase is likely due to enhanced NO production by the endothelium.

In the present study, there were no changes in plasma GPx activity or total antioxidant status. Plasma GPx is predominantly extracellular GPx (EC-GPx) and is primarily synthesized by the kidney (117). Plasma GPx levels are reduced in patients with renal impairment but the levels found in the present study are consistent with values in healthy individuals with normal renal function (118). The results of this study suggest that exercise training does not appear to have any effect on EC-GPx synthesis in the kidney.

Total antioxidant status is a measure of the ability of antioxidants in the plasma to inhibit oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenz-thiazoline sulphonate) by metmyoglobin after the addition of hydrogen peroxide. The results of the present study show that 12-weeks of endurance exercise training in patients with CAD does not alter total antioxidant status. Because the activity of SOD is not a factor in total antioxidant status the finding that total antioxidant status is unchanged following exercise training does not contradict the finding that plasma SOD activity was increased. Additionally, the fact that total antioxidant status did not change also suggests that there were no significant changes in dietary consumption of antioxidants.

The present study also demonstrated a 17% reduction in 8-isoprostane-$F_{2\alpha}$ (8-ISO) following 12-weeks of endurance exercise training in patients with CAD. 8-ISO has been shown to be associated with cardiovascular risk factors such as hypercholestrolemia (119) and hyperhomocysteinemia (120). The increase in plasma SOD activity likely accounts for the reduction in 8-ISO found in the present study. However, the increase in NO may also play a role. NO not only produces vasodilation but has potent antiatherogenic properties as well. NO inhibits platelet activation, vascular

smooth muscle cell proliferation and adhesion molecule expression and acts as an antioxidant (121,122). The reaction of NO with superoxide inhibits the formation of H_2O_2 (122). Therefore, the increased levels of NO found in the present study may also aid in scavenging superoxide. Additionally, NO is a chain-breaking antioxidant in the formation of lipid hydroperoxides (122). NO reacts 10,000 times faster with lipid hydroperoxides than α-tocopherol (122). Therefore, increased levels of NO may inhibit lipid peroxidation and spare α-tocopherol.

Recently it has been reported that acetylcholine-induced vasodilation and the effect of vitamin C on acetylcholine-induced vasodilation are both independent predictors of cardiovascular events in patients with CAD (20). The authors concluded that oxidative stress may contribute not only to endothelial dysfunction but also to coronary artery disease activity (20). Therefore, the exercise induced improvements in both endothelial function and oxidative stress in the present study may lead to a reduction in future cardiovascular events in CAD patients.

Inflammation

It is now established that CAD is an inflammatory process and future cardiovascular events can be predicted based on selected inflammatory markers. C-reactive protein (CRP) is the most robust of these markers and interleukin-6 (IL-6) has also shown some promise as a predictor of cardiovascular events (12). We found that CRP levels are reduced (33%) in CAD patients following 12 weeks of exercise training. However, IL-6 was not significantly reduced. The findings of the present study are consistent with previous findings from our laboratory. Previously, we had studied 11 CAD patients participating in standard cardiac rehabilitation. We found a significant

decrease in CRP and a trend (p=0.1) toward a reduction in IL-6. Prior to studies conducted in our lab, there had only been one cross sectional study investigating the relationship between exercise and CRP (13). In this study physical activity was associated with lower CRP levels (13). Because CRP has been shown to be an independent predictor of cardiovascular events in both healthy individuals and CAD patients one would hope that an intervention reducing CRP levels would reduce risk for future events as well. Aspirin therapy and statin therapy have both been shown to reduce both CRP and mortality (91,92). Although these medications have positive effects unto themselves their effectiveness may also be related to their anti-inflammatory actions as well (12). To date, however, there have been no investigations into whether a reduction in CRP affords a concomitant reduction in risk. At this time the only conclusions we can make regards to CRP is that 12-weeks of endurance exercise training appears to reduce plasma CRP levels. Whether these changes result in a reduction in risk of future cardiovascular events remains to be elucidated.

Conclusions

The present study was designed to determine if 12-weeks of endurance exercise training had a positive effect on endothelial function and arterial stiffness and if so attempt to elucidate possible mechanisms. This study demonstrates that endurance exercise training in CAD improves endothelial function and reduces arterial stiffness. Parallel to these findings we also demonstrated increased plasma NOx and plasma SOD activity and reductions in 8-ISO and CRP. These results suggest that there is an increased production of NO and a reduction in oxidative stress and inflammation in CAD following 12-weeks of endurance training. The results of this study add to the evidence

that exercise training is beneficial for CAD patients by helping elucidate the possible mechanisms for improvements seen with exercise training in these patients.

Limitations

This study was designed to test the effectiveness of 12-weeks of endurance exercise training as part of a cardiac rehabilitation program. The multidisciplinary approach to cardiac rehabilitation may confound the results of this study. Behavioral and dietary changes as a result of counseling could also play a role in these improvements. It is our belief, however, that because exercise training is the primary component of cardiac rehabilitation it is responsible for the changes seen in the present study. The results of the present study have real life implications because we have demonstrated that the standard of care for patients with CAD results in improved vascular biology.

The interpretation of the results of the present study may be limited due to small sample sizes. It is possible that changes that occurred in our sample are the result of selection bias or are simply outliers and changes would not have been found had larger sample sizes been used. More likely though, a larger sample size would result in the same findings only with greater significance.

REFERENCES

1. Suwaidi JA, Hamasaki S, Higano ST, Nishimura RA, Holmes DR, Jr., Lerman A. Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction. Circulation 2000;101:948-54.

2. Schachinger V, Zeiher AM. Prognostic implications of endothelial dysfunction: does it mean anything? Coron Artery Dis 2001;12:435-43.

3. Hambrecht R, Wolf A, Gielen S, et al. Effect of exercise on coronary endothelial function in patients with coronary artery disease. N Engl J Med 2000;342:454-60.

4. Kingwell BA. Large artery stiffness: implications for exercise capacity and cardiovascular risk. Clin Exp Pharmacol Physiol 2002;29:214-7.

5. Ohara Y, Peterson TE, Harrison DG. Hypercholesterolemia increases endothelial superoxide anion production. J Clin Invest 1993;91:2546-51.

6. Clarkson P, Montgomery HE, Mullen MJ, et al. Exercise training enhances endothelial function in young men. J Am Coll Cardiol 1999;33:1379-85.

7. Varin R, Mulder P, Richard V, et al. Exercise improves flow-mediated vasodilatation of skeletal muscle arteries in rats with chronic heart failure. Role of nitric oxide, prostanoids, and oxidant stress. Circulation 1999;99:2951-7.

8. Gokce N, Vita JA, Bader DS, et al. Effect of exercise on upper and lower extremity endothelial function in patients with coronary artery disease. Am J Cardiol 2002;90:124-7.

9. Feske W, Finkelstein SM, Francis G, Cohn JN. Arterial vascular compliance response to exercise in hypertension. Biomed Sci Instrum 1988;24:161-5.

10. Eugene M, Vandewalle H, Bertholon JF, Teillac A. Arterial elasticity and physical working capacity in young men. J Appl Physiol 1986;61:1720-3.

11. Tarnawski M, Cybulski G, Doorly D, Dumoulin C, Darrow R, Caro C. Noninvasive determination of local wavespeed and distensibility of the femoral artery by comb-excited Fourier velocity-encoded magnetic resonance imaging: measurements on athletic and nonathletic human subjects. Heart Vessels 1994;9:194-201.

12. Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 2000;342:836-43.

13. Geffken D, Cushman M, Burke G, Polak J, Sakkinen P, Tracy R. Association between physical activity and markers of inflammation in a healthy elderly population. Am J Epidemiol 2001;153:242-50.

14. Pepine CJ. Clinical implications of endothelial dysfunction. Clin Cardiol 1998;21:795-9.

15. Hambrecht R, Fiehn E, Weigl C, et al. Regular physical exercise corrects endothelial dysfunction and improves exercise capacity in patients with chronic heart failure. Circulation 1998;98:2709-15.

16. Higashi Y, Sasaki S, Kurisu S, et al. Regular aerobic exercise augments endothelium-dependent vascular relaxation in normotensive as well as hypertensive subjects: role of endothelium-derived nitric oxide. Circulation 1999;100:1194-202.

17. Drexler H, Hornig B. Endothelial dysfunction in human disease. J Mol Cell Cardiol 1999;31:51-60.

18. Delp MD, Laughlin MH. Time course of enhanced endothelium-mediated dilation in aorta of trained rats. Med Sci Sports Exerc 1997;29:1454-61.

19. Jungersten L, Ambring A, Wall B, Wennmalm A. Both physical fitness and acute exercise regulate nitric oxide formation in healthy humans. J Appl Physiol 1997;82:760-4.

20. Heitzer T, Schlinzig T, Krohn K, Meinertz T, Munzel T. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001;104:2673-8.

21. Gokce N, Keaney JF, Jr., Frei B, et al. Long-term ascorbic acid administration reverses endothelial vasomotor dysfunction in patients with coronary artery disease. Circulation 1999;99:3234-40.

22. Landmesser U, Merten R, Spiekermann S, Buttner K, Drexler H, Hornig B. Vascular extracellular superoxide dismutase activity in patients with coronary artery disease: relation to endothelium-dependent vasodilation. Circulation 2000;101:2264-70.

23. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. J Clin Invest 2000;105:1631-9.

24. Furchgott RF, Zawadzki JV. The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. Nature 1980;288:373-6.

25. Palmer RM, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. Nature 1987;327:524-6.

26. Ignarro LJ, Buga GM, Wood KS, Byrns RE, Chaudhuri G. Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. Proc Natl Acad Sci U S A 1987;84:9265-9.

27. Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329:2002-12.

28. Shaul PW, Smart EJ, Robinson LJ, et al. Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. J Biol Chem 1996;271:6518-22.

29. Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. J Biol Chem 1996;271:22810-4.

30. Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? J Clin Invest 1997;100:2146-52.

31. Pritchard KA, Jr., Ackerman AW, Gross ER, et al. Heat shock protein 90 mediates the balance of nitric oxide and superoxide anion from endothelial nitric-oxide synthase. J Biol Chem 2001;276:17621-4.

32. Dillon GA, Vita JA. Nitric oxide and endothelial dysfunction. In: Loscalzo J, Vita JA, eds. Nitric oxide and the cardiovascular system. Totawa, NJ: Humana Press, 2000:207-226.

33. Vasquez-Vivar J, Kalyanaraman B, Martasek P, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. Proc Natl Acad Sci U S A 1998;95:9220-5.

34. Moncada S, Palmer RM, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991;43:109-42.

35. Cohen RA. Role of nitric oxide in vasomotor control. In: Loscalzo J, Vita JA, eds. Nitric oxide and the cardiovascular system. Totowa, NJ: Humana Press, 2000:105-122.

36. Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, Cohen RA. Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. Nature 1994;368:850-3.

37. Martin W, Furchgott RF, Villani GM, Jothianandan D. Depression of contractile responses in rat aorta by spontaneously released endothelium-derived relaxing factor. J Pharmacol Exp Ther 1986;237:529-38.

38. Schmidt HH, Pollock JS, Nakane M, Forstermann U, Murad F. Ca2+/calmodulin-regulated nitric oxide synthases. Cell Calcium 1992;13:427-34.

39. Vallance P, Chan N. Endothelial function and nitric oxide: clinical relevance. Heart 2001;85:342-50.

40. Jacobs ER, Cheliakine C, Gebremedhin D, Birks EK, Davies PF, Harder DR. Shear activated channels in cell-attached patches of cultured bovine aortic endothelial cells. Pflugers Arch 1995;431:129-31.

41. Fleming I, Bauersachs J, Fisslthaler B, Busse R. Ca2+-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. Circ Res 1998;82:686-95.

42. Fisslthaler B, Dimmeler S, Hermann C, Busse R, Fleming I. Phosphorylation and activation of the endothelial nitric oxide synthase by fluid shear stress. Acta Physiol Scand 2000;168:81-8.

43. Nishida K, Harrison DG, Navas JP, et al. Molecular cloning and characterization of the constitutive bovine aortic endothelial cell nitric oxide synthase. J Clin Invest 1992;90:2092-6.

44. Sessa WC, Pritchard K, Seyedi N, Wang J, Hintze TH. Chronic exercise in dogs increases coronary vascular nitric oxide production and endothelial cell nitric oxide synthase gene expression. Circ Res 1994;74:349-53.

45. Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2, and prostacyclin. Pharmacol Rev 1978;30:293-331.

46. Lamontagne D, Konig A, Bassenge E, Busse R. Prostacyclin and nitric oxide contribute to the vasodilator action of acetylcholine and bradykinin in the intact rabbit coronary bed. J Cardiovasc Pharmacol 1992;20:652-7.

47. Mombouli JV, Bissiriou I, Agboton V, Vanhoutte PM. Endothelium-derived hyperpolarizing factor: a key mediator of the vasodilator action of bradykinin. Immunopharmacology 1996;33:46-50.

48. Feletou M, Vanhoutte PM. Endothelium-derived hyperpolarizing factor. Clin Exp Pharmacol Physiol 1996;23:1082-90.

49. Feletou M, Vanhoutte PM. Endothelium-dependent hyperpolarization of canine coronary smooth muscle. Br J Pharmacol 1988;93:515-24.

50. Yanagisawa M, Kurihara H, Kimura S, Goto K, Masaki T. A novel peptide vasoconstrictor, endothelin, is produced by vascular endothelium and modulates smooth muscle Ca2+ channels. J Hypertens Suppl 1988;6:S188-91.

51. Inoue A, Yanagisawa M, Kimura S, et al. The human endothelin family: three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci U S A 1989;86:2863-7.

52. Xu D, Emoto N, Giaid A, et al. ECE-1: a membrane-bound metalloprotease that catalyzes the proteolytic activation of big endothelin-1. Cell 1994;78:473-85.

53. Duchman SM, Thohan V, Kalra D, Torre-Amione G. Endothelin-1: a new target of therapeutic intervention for the treatment of heart failure. Curr Opin Cardiol 2000;15:136-40.

54. Coleman RA, Smith WL, Narumiya S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. Pharmacol Rev 1994;46:205-29.

55. Mombouli JV, Vanhoutte PM. Endothelial dysfunction: from physiology to therapy. J Mol Cell Cardiol 1999;31:61-74.

56. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res 2000;87:840-4.

57. Munzel T, Harrison DG. Increased superoxide in heart failure: a biochemical baroreflex gone awry. Circulation 1999;100:216-8.

58. Palombo C, Lubrano V, Sampietro T. Oxidative stress, F2-isoprostanes and endothelial dysfunction in hypercholesterolemia. Cardiovasc Res 1999;44:474-6.

59. Nichols WW, O'Rourke MF. McDonald's blood flow in arteries: Theoretical, experimental and clinical principles. 4th ed. London: Arnold, 1998.

60. Kelly RP, Tunin R, Kass DA. Effect of reduced aortic compliance on cardiac efficiency and contractile function of in situ canine left ventricle. Circ Res 1992;71:490-502.

61. Elzinga G, Westerhof N. Pressure and flow generated by the left ventricle against different impedances. Circ Res 1973;32:178-86.

62. Sakai S, Miyauchi T, Kobayashi M, Yamaguchi I, Goto K, Sugishita Y. Inhibition of myocardial endothelin pathway improves long-term survival in heart failure. Nature 1996;384:353-5.

63. Kelly RP, Ting CT, Yang TM, et al. Effective arterial elastance as index of arterial vascular load in humans. Circulation 1992;86:513-21.

64. Nichols WW, Edwards DG. Arterial elastance and wave reflection augmentation of systolic blood pressure: deleterious effects and implications for therapy. J Cardiovasc Pharmacol Ther 2001;6:5-21.

65. Bramwell JC, Hill AV. Velocity of transmission of the pulse wave and elasticity of arteries. Lancet 1922;1:891-892.

66. Bramwell JC, Hill AV, McSwiney BA. The velocity of the pulse wave in man in relation to age as studied by the hotwire sphygmograph. Heart 1923;10:233-249.

67. Murgo JP, Westerhof N, Giolma JP, Altobelli SA. Manipulation of ascending aortic pressure and flow wave reflections with the Valsalva maneuver: relationship to input impedance. Circulation 1981;63:122-32.

68. Kirkpatrick RD, Campbell KB, Bell DL, Taheri H. Method for studying arterial wave transmission effects on left ventricular function. Am J Physiol 1991;260:H1003-12.

69. Murgo JP, Westerhof N, Giolma JP, Altobelli SA. Aortic input impedance in normal man: relationship to pressure wave forms. Circulation 1980;62:105-16.

70. Drexler H, Hayoz D, Munzel T, et al. Endothelial function in chronic congestive heart failure. Am J Cardiol 1992;69:1596-601.

71. Rubanyi GM, Romero JC, Vanhoutte PM. Flow-induced release of endothelium-derived relaxing factor. Am J Physiol 1986;250:H1145-9.

72. Cooke JP, Singer AH, Tsao P, Zera P, Rowan RA, Billingham ME. Antiatherogenic effects of L-arginine in the hypercholesterolemic rabbit. J Clin Invest 1992;90:1168-72.

73. Rossitch E, Jr., Alexander E, 3rd, Black PM, Cooke JP. L-arginine normalizes endothelial function in cerebral vessels from hypercholesterolemic rabbits. J Clin Invest 1991;87:1295-9.

74. Gatzka CD, Cameron JD, Kingwell BA, Dart AM. Relation between coronary artery disease, aortic stiffness, and left ventricular structure in a population sample. Hypertension 1998;32:575-8.

75. Watanabe H, Ohtsuka S, Kakihana M, Sugishita Y. Coronary circulation in dogs with an experimental decrease in aortic compliance. J Am Coll Cardiol 1993;21:1497-506.

76. Saeki A, Recchia F, Kass DA. systolic flow augmentation in hearts ejecting into a model of stiff aging vasculature. Influence on myocardial perfusion-demand balance. Circ Res 1995;76:132-41.

77. Kass DA, Saeki A, Tunin RS, Recchia FA. Adverse influence of systemic vascular stiffening on cardiac dysfunction and adaptation to acute coronary occlusion. Circulation 1996;93:1533-41.

78. Fitch RM, Vergona R, Sullivan ME, Wang Y. Nitric oxide synthase inhibition increases aortic stiffness measured by pulse wave velocity in rats. Cardiovasc Res 2001;51:351-8.

79. Weinberg PD, Habens F, Kengatharan M, et al. Characteristics of the pulse waveform during altered nitric oxide synthesis in the rabbit. Br J Pharmacol 2001;133:361-70.

80. Kass DA, Shapiro EP, Kawaguchi M, et al. Improved arterial compliance by a novel advanced glycation end-product crosslink breaker. Circulation 2001;104:1464-70.

81. Williams IL, Wheatcroft SB, Shah AM, Kearney MT. Obesity, atherosclerosis and the vascular endothelium: mechanisms of reduced nitric oxide bioavailability in obese humans. Int J Obes Relat Metab Disord 2002;26:754-64.

82. Vidal F, Colome C, Martinez-Gonzalez J, Badimon L. Atherogenic concentrations of native low-density lipoproteins down- regulate nitric-oxide-synthase mRNA and protein levels in endothelial cells. Eur J Biochem 1998;252:378-84.

83. Landmesser U, Harrison DG. Oxidant stress as a marker for cardiovascular events: Ox marks the spot. Circulation 2001;104:2638-40.

84. Bridges AB, Scott NA, Pringle TH, McNeill GP, Belch JJ. Relationship between the extent of coronary artery disease and indicators of free radical activity. Clin Cardiol 1992;15:169-74.

85. Bauersachs J, Bouloumie A, Fraccarollo D, Hu K, Busse R, Ertl G. Endothelial dysfunction in chronic myocardial infarction despite increased vascular endothelial nitric oxide synthase and soluble guanylate cyclase expression: role of enhanced vascular superoxide production. Circulation 1999;100:292-8.

86. Kojda G, Harrison D. Interactions between NO and reactive oxygen species: pathophysiological importance in atherosclerosis, hypertension, diabetes and heart failure. Cardiovasc Res 1999;43:562-71.

87. Higashi Y, Sasaki S, Nakagawa K, et al. A comparison of angiotensin-converting enzyme inhibitors, calcium antagonists, beta-blockers and diuretic agents on reactive hyperemia in patients with essential hypertension: a multicenter study. J Am Coll Cardiol 2000;35:284-91.

88. Karlsson K, Sandstrom J, Edlund A, Edlund T, Marklund SL. Pharmacokinetics of extracellular-superoxide dismutase in the vascular system. Free Radic Biol Med 1993;14:185-90.

89. Inoue N, Ramasamy S, Fukai T, Nerem RM, Harrison DG. Shear stress modulates expression of Cu/Zn superoxide dismutase in human aortic endothelial cells. Circ Res 1996;79:32-7.

90. Ridker PM, Buring JE, Rifai N. Soluble P-selectin and the risk of future cardiovascular events. Circulation 2001;103:491-5.

91. Ridker PM, Rifai N, Pfeffer MA, et al. Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events (CARE) Investigators. Circulation 1998;98:839-44.

92. Ridker PM, Cushman M, Stampfer MJ, Tracy RP, Hennekens CH. Inflammation, aspirin, and the risk of cardiovascular disease in apparently healthy men. N Engl J Med 1997;336:973-9.

93. DeSouza CA, Shapiro LF, Clevenger CM, et al. Regular aerobic exercise prevents and restores age-related declines in endothelium-dependent vasodilation in healthy men. Circulation 2000;102:1351-7.

94. Hornig B, Maier V, Drexler H. Physical training improves endothelial function in patients with chronic heart failure. Circulation 1996;93:210-4.

95. Linke A, Schoene N, Gielen S, et al. Endothelial dysfunction in patients with chronic heart failure: systemic effects of lower-limb exercise training. J Am Coll Cardiol 2001;37:392-7.

96. Lavrencic A, Salobir BG, Keber I. Physical training improves flow-mediated dilation in patients with the polymetabolic syndrome. Arterioscler Thromb Vasc Biol 2000;20:551-5.

97. Kingwell BA, Arnold PJ, Jennings GL, Dart AM. Spontaneous running increases aortic compliance in Wistar-Kyoto rats. Cardiovasc Res 1997;35:132-7.

98. Cameron JD, Dart AM. Exercise training increases total systemic arterial compliance in humans. Am J Physiol 1994;266:H693-701.

99. Vaitkevicius PV, Fleg JL, Engel JH, et al. Effects of age and aerobic capacity on arterial stiffness in healthy adults. Circulation 1993;88:1456-62.

100. Kingwell BA, Arnold PJ, Jennings GL, Dart AM. The effects of voluntary running on cardiac mass and aortic compliance in Wistar-Kyoto and spontaneously hypertensive rats. J Hypertens 1998;16:181-5.

101. Tanaka H, Dinenno FA, Monahan KD, Clevenger CM, DeSouza CA, Seals DR. Aging, habitual exercise, and dynamic arterial compliance. Circulation 2000;102:1270-5.

102. Sun D, Huang A, Koller A, Kaley G. Short-term daily exercise activity enhances endothelial NO synthesis in skeletal muscle arterioles of rats. J Appl Physiol 1994;76:2241-7.

103. Powers SK, Ji LL, Leeuwenburgh C. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med Sci Sports Exerc 1999;31:987-97.

104. Jenkins RR. Free radical chemistry. Relationship to exercise. Sports Med 1988;5:156-70.

105. Rush JW, Laughlin MH, Woodman CR, Price EM. SOD-1 expression in pig coronary arterioles is increased by exercise training. Am J Physiol Heart Circ Physiol 2000;279:H2068-76.

106. Sarabi M, Fugmann A, Karlstrom B, Berne C, Lithell H, Lind L. An ordinary mixed meal transiently impairs endothelium-dependent vasodilation in healthy subjects. Acta Physiol Scand 2001;172:107-13.

107. Vogel RA, Corretti MC, Plotnick GD. A comparison of brachial artery flow-mediated vasodilation using upper and lower arm arterial occlusion in subjects with and without coronary risk factors. Clin Cardiol 2000;23:571-5.

108. Hornig B, Arakawa N, Kohler C, Drexler H. Vitamin C improves endothelial function of conduit arteries in patients with chronic heart failure. Circulation 1998;97:363-8.

109. Kubo SH, Rector TS, Bank AJ, Williams RE, Heifetz SM. Endothelium-dependent vasodilation is attenuated in patients with heart failure. Circulation 1991;84:1589-96.

110. Karamanoglu M, O'Rourke MF, Avolio AP, Kelly RP. An analysis of the relationship between central aortic and peripheral upper limb pressure waves in man. Eur Heart J 1993;14:160-7.

111. Wilkinson IB, Fuchs SA, Jansen IM, et al. Reproducibility of pulse wave velocity and augmentation index measured by pulse wave analysis. J Hypertens 1998;16:2079-84.

112. Oyanagui Y. Reevaluation of assay methods and establishment of kit for superoxide dismutase activity. Anal Biochem 1984;142:290-6.

113. Flohe L, Gunzler WA. Assays of glutathione peroxidase. Methods Enzymol 1984;105:114-21.

114. Cameron JD, Rajkumar C, Kingwell BA, Jennings GL, Dart AM. Higher systemic arterial compliance is associated with greater exercise time and lower blood pressure in a young older population. J Am Geriatr Soc 1999;47:653-6.

115. Wilkinson IB, Qasem A, McEniery CM, Webb DJ, Avolio AP, Cockcroft JR. Nitric oxide regulates local arterial distensibility in vivo. Circulation 2002;105:213-7.

116. Diaz MN, Frei B, Vita JA, Keaney JF, Jr. Antioxidants and atherosclerotic heart disease. N Engl J Med 1997;337:408-16.

117. Yoshimura S, Watanabe K, Suemizu H, et al. Tissue specific expression of the plasma glutathione peroxidase gene in rat kidney. J Biochem (Tokyo) 1991;109:918-23.

118. Yoshimura S, Suemizu H, Nomoto Y, et al. Plasma glutathione peroxidase deficiency caused by renal dysfunction. Nephron 1996;73:207-11.

119. Reilly MP, Pratico D, Delanty N, et al. Increased formation of distinct F2 isoprostanes in hypercholesterolemia. Circulation 1998;98:2822-8.

120. Voutilainen S, Morrow JD, Roberts LJ, 2nd, et al. Enhanced in vivo lipid peroxidation at elevated plasma total homocysteine levels. Arterioscler Thromb Vasc Biol 1999;19:1263-6.

121. Ortega Mateo A, Amaya Aleixandre de A. Nitric oxide reactivity and mechanisms involved in its biological effects. Pharmacol Res 2000;42:421-7.

122. Patel RP, Levonen A, Crawford JH, Darley-Usmar VM. Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. Cardiovasc Res 2000;47:465-74.

BIOGRAPHICAL SKETCH

David George Edwards completed his undergraduate training at the University of Delaware in 1991 with a major in Physical Education Studies with an emphasis in exercise science. He entered graduate school at Wake Forest University where he received his master of science degree in Health & exercise Science in 1993. His advisor was Dr. Stephen Messier. At Wake Forest he worked in the Wake Forest Runner's Clinic as well as the Wake Forest Cardiac Rehabilitation program with Dr. Peter Brubaker and Dr. Henry Miller. Following graduation he worked in pharmaceutical clinical research and then returned to Wake Forest to work with Dr. Michael Berry on an NIH funded research study. In the fall of 1998, he entered the doctoral program at the University of Florida in the Department of Exercise and Sport Science under the direction of Dr. Randy Braith. David served part-time as an adjunct faculty member at Santa Fe Community College, teaching in the Cardiopulmonary Technology Program. He also worked as an Exercise Physiologist in the Shands Cardiac Rehabilitation Program. David has taken a position as an Assistant Professor at the University of New Hampshire beginning in the fall of 2002.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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